MECHANISM OF ACTION OF AN ANTIFUNGAL ANTIBIOTIC, RI-331, (S) 2-AMINO-4-OXO-5-HYDROXYPENTANOIC ACID; KINETICS OF INACTIVATION OF HOMOSERINE DEHYDROGENASE
FROM *Saccharomyces cerevisiae*

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An antifungal antibiotic (S) 2-amino-4-oxo-5-hydroxypentanoic acid, inhibited the biosynthesis of the aspartate family of amino acids (methionine, isoleucine and threonine) followed by the inhibition of protein biosynthesis in *Saccharomyces cerevisiae*. This inhibition was effected by impeding the biosynthesis of their common intermediate precursor, homoserine. The inhibition of biosynthesis of homoserine by the antibiotic was attributable to inactivation of homoserine dehydrogenase [EC 1.1.1.3], which is involved in the conversion of aspartate semialdehyde to homoserine in the metabolic pathway leading to threonine, methionine and isoleucine. Since such enzymic activity is not present in animal cells, the selective antifungal activity of the antibiotic is thus explained.

The recent increase of opportunistic fungal infection prompted us to search for new types of antifungal agents. In our program for screening of antifungal agents, we isolated an amino acid analog, (S) 2-amino-4-oxo-5-hydroxypentanoic acid (Fig. 1) coded as RI-331, from a culture broth of *Streptomyces* sp. The antibiotic had *in vitro* activity against several pathogenic fungi of medical importance, including *Candida albicans* and *Cryptoccocus neoformans*, and was effective in the treatment of systemic murine candidiasis being highly tolerated in mice[1]. Our major concern was to understand the molecular basis of the selective antifungal action of the antibiotic. Our initial studies with growing cells of *Saccharomyces cerevisiae* showed that RI-331 preferentially lowered the cellular level of three aspartate family amino acid (*viz* threonine, methionine and isoleucine). Also the antifungal action of RI-331 was antagonized by these amino acids and, to a greater extent, by their metabolic precursor homoserine, suggesting that the site of action of the antibiotic may be on the metabolic step(s) involved in the biosynthesis of homoserine or its precursor[2,3]. This hypothesis was confirmed by the results of studies with the enzymatic system prepared from yeast cells; homoserine dehydrogenase involved in the conversion of aspartate semialdehyde to homoserine was significantly inhibited by the antibiotic[4].

In this publication the kinetics of this inhibition of homoserine dehydrogenase by the antibiotic are described.

Fig. 1. Structure of RI-331.
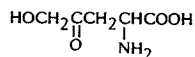
$$HOCH_2\underset{\underset{O}{\|}}{C}CH_2\underset{\underset{NH_2}{|}}{C}HCOOH$$

Materials and Methods

Yeast Strains, Media and Cultivation

Several mutants of *Saccharomyces cerevisiae*, blocked in the synthesis of homoserine from aspartate, were used. Two such strains, S2614C (*hom2 aro*1C *lys*1 *ade*1 *try*1 *arg*4-1 *gal*2) lacking aspartate semialdehyde dehydrogenase [EC 1.2.1.11] and STX25-2A (*hom*6 *ade*6 *his*4 *ura*1 *gal*2) lacking homoserine dehydrogenase were kindly given by the Yeast Genetic Stock Center, University of California, Berkeley, CA. These strains were cultured in YPD broth (yeast extract 1%, peptone 2% and glucose 2%) aerobically at 27°C.

Preparation of Enzymes

Aspartate kinase was purified from either S2614C or STX25-2A mutant strain by the method as described by BLACK *et al.*[5]. Aspartate semialdehyde dehydrogenase was purified from the *hom*6 mutant, STX25-2A (lacking homoserine dehydrogenase), and homoserine dehydrogenase was purified from the *hom*2 mutant, S2614C (lacking aspartate semialdehyde dehydrogenase) as described elsewhere[6,7]. The enzymes were further purified by column chromatography of DEAE cellulose (DE52) and then hydroxyapatite.

Enzyme Reactions

Aspartate kinase activity was determined by measuring the rate of forward reaction. The reaction mixture contained 100 mM Tris-HCl, pH 8.0, 100 mM L-aspartic acid, 20 mM ATP, 20 mM $MgCl_2$, 400 mM hydroxylamine hydrochloride and 200 µg of aspartate kinase in a final volume of 1 ml. Incubation was at 27°C for 30 minutes, in which aspartylhydroxamate is formed by the interaction of aspartylphosphate with hydroxylamine. Then, 1 ml of 5% $FeCl_3 \cdot 6H_2O$ dissolved in 0.1 N HCl was added and the absorbance of aspartylhydroxamate-iron complex formed was measured at 540 nm[5].

Aspartate semialdehyde dehydrogenase activity coupled with aspartate kinase and aspartate semialdehyde dehydrogenase was measured in the forward reaction by following NADPH disappearance monitored by the decrease of absorbance at 340 nm. The reaction mixture contained 100 mM Tris-HCl, pH 8.0, 100 mM L-aspartic acid, 20 mM ATP, 20 mM $MgCl_2$, 0.08 mM NADPH, aspartate kinase 100 µg and aspartate semialdehyde dehydrogenase 20 µg in a total volume of 1 ml. Incubation was at 27°C.

Homoserine dehydrogenase activity in the forward reaction was measured by NADPH disappearance monitored by reduction of absorbance at 340 nm. The reaction mixture contained 100 mM potassium phosphate buffer pH 6.7, 0.08 mM NADPH, 10 µg of homoserine dehydrogenase and 0.1 mM aspartate semialdehyde was incubated at 27°C.

Homoserine dehydrogenase activity in the reverse reaction was measured by NADPH production corresponding to the increase of absorbance at 340 nm. The reaction mixture contained 100 mM Tris-HCl, pH 9.0, 0.2 mM NADP, 10 µg homoserine dehydrogenase, and 100 mM homoserine. Incubation was at 27°C.

Chemicals: L-Aspartic acid, L-aspartylhydroxamate, L-homoserine, hydroxylamine-HCl, ATP, NADP and NADPH were purchased from Sigma Chemical Co. Ltd., St. Louis, MO. RI-331 and aspartate semialdehyde were kindly supplied by Taisho Pharmaceutical Co. Ltd, Ohmiya City, Saitama prefecture, Japan.

Results

Effect of RI-331 on Aspartate Kinase and Aspartate
Semialdehyde Dehydrogenase

The aspartate kinase activity of strains S2614C or STX25-2A was not inhibited by RI-331 at 5 mM (Table 1), nor was the aspartate semialdehyde dehydrogenase involved in converting aspartylphosphate to aspartate semialdehyde, significantly affected by the antibiotic at such high concentration (Table 2).
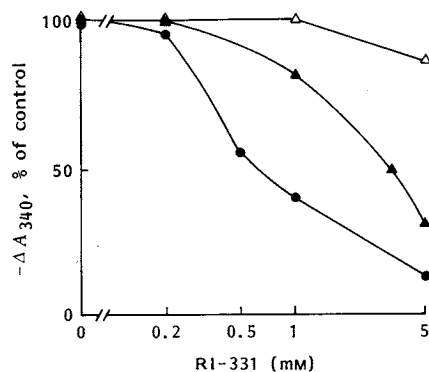
Effect of RI-331 on Homoserine Dehydrogenase

Homoserine dehydrogenase activity in the forward reaction, determined by NADPH dehydrogenation depending on added substrate aspartate semialdehyde (ASA), was significantly inhibited by the antibiotic. The inhibition of the enzyme activity was enhanced, if NADP was added to the reaction mixture at high

Table 1.   The activity of aspartate kinase from *Saccharomyces cerevisiae* S2614C (*hom2*) lacking aspartate semialdehyde dehydrogenase was measured.

	A_{540}
Complete	0.45
$-$Asp	0
$-$ATP	0.04
$+$RI-331 5 mM	0.44
$+$RI-331 1 mM	0.48

Table 2.   Aspartate semialdehyde dehydrogenase activity was determined by coupling reaction of both aspartate kinase and aspartate semialdehyde dehydrogenase from *Saccharomyces cerevisiae* STX25-2A (*hom6*) lacking homoserine dehydrogenase.

	$-\Delta A_{340}$
Complete	0.21
$-$Asp	0.04
$-$ASADH	0.03
$+$RI-331 10 mM	0.21
$+$RI-331 1 mM .	0.22

Fig. 2.   Influence of NADP concentration on the inhibition of homoserine dehydrogenase activity in the forward reaction by RI-331.

$\triangle$ $-$NADP, $\blacktriangle$ $+$NADP 0.2 mM, $\bullet$ $+$NADP 0.8 mM.

Control; no addition of RI-331, expressed by 100%.
Incubation was at 27°C for 10 minutes.

Fig. 3.   Lineweaver-Burk plot with respect to substrate aspartate semialdehyde of the inhibition by RI-331 of homoserine dehydrogenase in the forward reaction in the presence of 0.2 mM NADP.

$\bigcirc$ $+$RI-331 5 mM, $\blacktriangle$ $+$RI-331 1 mM, $\bullet$ no addition of RI-331.

$1/v$; mmol$^{-1} \cdot 30$ minutes $\cdot$ mg.

concentrations relative to NADPH (Fig. 2). NADP was added to the reaction mixture despite not being required in the forward reaction, but the addition of NADP was essential for exhibition of inhibition of the forward reaction by the antibiotic. The rate of the forward reaction of control (without RI-331) was not affected by addition of NADP. These results presumably indicate that RI-331 may interact with the enzyme-NADP complex and then enzyme inhibition take place. The inhibition was the mixed type of competitive and noncompetitive inhibition with respect to ASA with a Ki value of 2 mM vs. a Km value of 0.05 mM at a concentration of NADP 0.2 mM, and the reaction was inhibited at a high concentration of the substrate ASA itself more than 0.04 mM (substrate inhibition) (Fig. 3). The inhibition of this forward reaction by the antibiotic was noncompetitive with respect to NADP, and the association constant for NADP in the presence of the antibiotic revealed two values of 0.4 and 2 mM, respectively, as analyzed by Dixon-plot (Fig. 4), suggesting that the enzyme might interact with the antibiotic in two ways to give tertiary complexes (enzyme, NADP and antibiotic) of differing stabilities.

Homoserine dehydrogenase was more significantly inhibited by the antibiotic in the reverse reaction than in the forward reaction. The inhibition was competitive with respect to the substrate homoserine showing a Ki value of 0.025 mM vs. Km value of 17 mM (Fig. 5). At a physiological concentration of
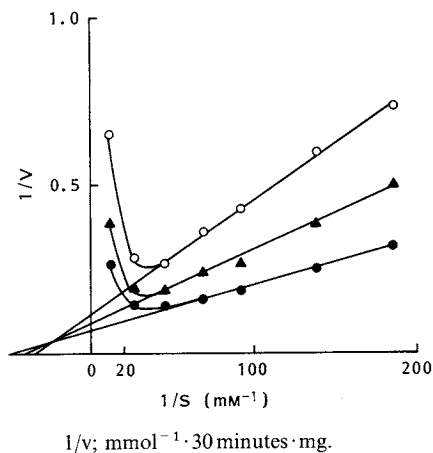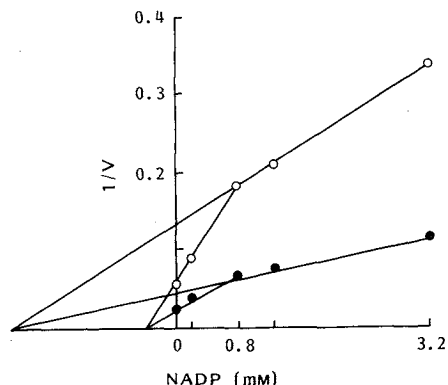
Fig. 4. Dixon-plot with respect to NADP of the inhibition of homoserine dehydrogenase in the forward reaction by RI-331.

○ +RI-331 5 mM, ● +RI-331 1 mM.

Initial velocity of the forward reaction was plotted versus varing concentrations of NADP in the presence of RI-331, 1 and 5 mM, respectively. $1/v$; $mmol^{-1} \cdot 30$ minutes·mg.

Fig. 5. Lineweaver-Burk plot with respect to substrate homoserine of the inhibition of homoserine dehydrogenase in the reverse reaction by RI-331.
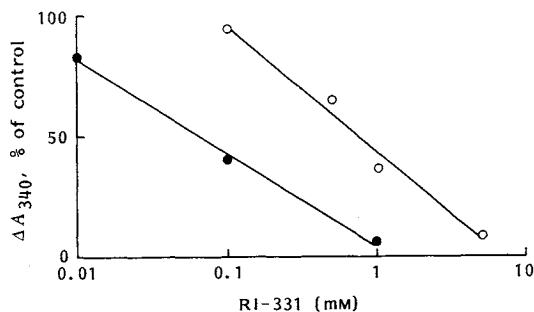
○ +RI-331 1 mM, ▲ +RI-331 0.1 mM, ● no addition of RI-331.
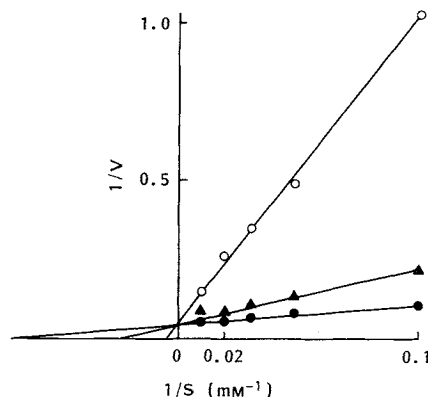
$1/v$; $\mu mol^{-1} \cdot 10$ minutes·mg.

Fig. 6. Susceptibility to RI-331 of homoserine dehydrogenase at physiological concentration of NADP in reverse (A) and forward (B) reactions.
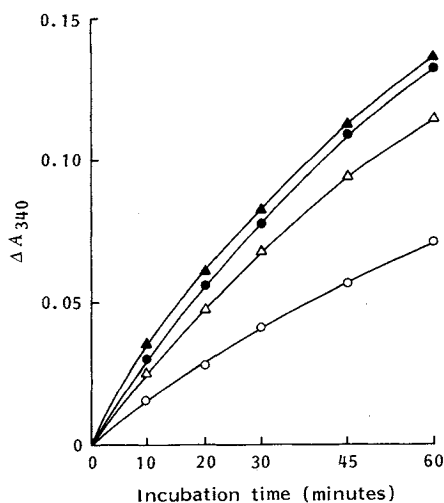
● A, ○ B.

NADP concentration was adjusted to 0.2 mM. Incubation was at 27°C for 10 minutes in the forward (B) and 30 minutes in the reverse (A) reactions, respectively.

Fig. 7. Enhancement of RI-331-inhibitory effect on homoserine dehydrogenase in the reverse reaction by prior exposure to NADP.

▲ A, ● B, △ C, ○ D.

A; no addition of RI-331, B and C; NADP and homoserine were last added to the reaction mixture, respectively, D; enzyme, NADP, and RI-331 were mixed first, then buffer solution, and finally added by homoserine to start reaction. The final concentration of RI-331 (in B to D) was 0.01 mM, and NADP 0.1 mM (in A to D).

NADP 0.2 mM, the enzyme was at least 10 times more susceptible to the antibiotic in the reverse reaction than in the forward reaction (Fig. 6). Moreover, prior exposure of the enzyme to NADP before starting the reaction enhanced the extent of inhibition of the enzyme in the reverse reaction (Fig. 7).

Fig. 8. Scheme of enzyme-substrate complex in homoserine dehydrogenase reaction, and an assumed interaction of RI-331 with enzyme.
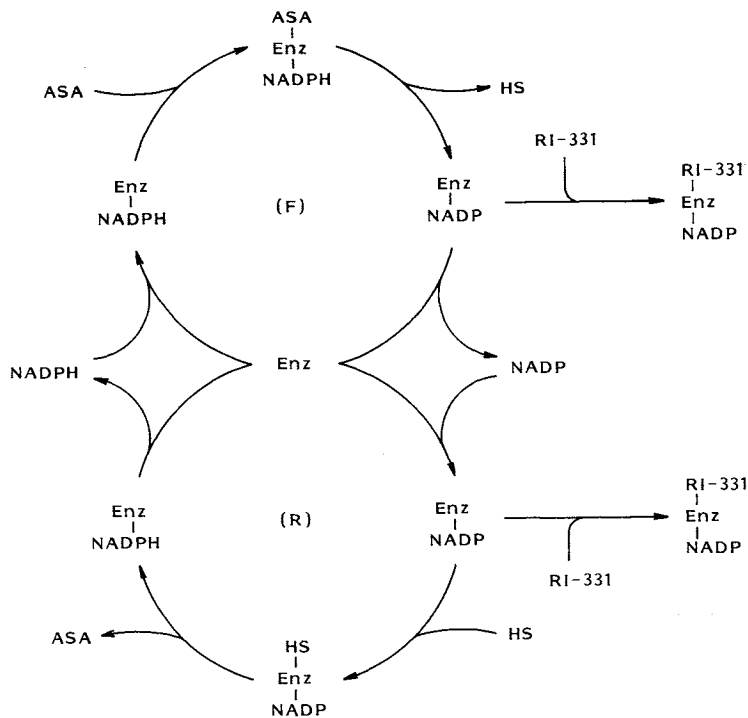
Abbreviations: Enz; homoserine dehydrogenase from *Saccharomyces cerevisiae*, ASA; Aspartate semialdehyde, HS; homoserine, (F); forward reaction, (R); reverse reaction.

Discussion

Our preceding hypothesis, that the antifungal activity of RI-331 is due to the inhibition of the biosynthesis of the aspartate family of amino acids including threonine, methionine and isoleucine, followed by the inhibition of protein synthesis[2,3], was confirmed with the demonstration of inhibition of homoserine biosynthesis by the antibiotic[4]. In the pathway from aspartate to homoserine, three enzymes (aspartate kinase, aspartate semialdehyde dehydrogenase and homoserine dehyrogenase) are involved. The former two enzymes, aspartate kinase and aspartate semialdehyde dehydrogenase were refractory to the antibiotic (Tables 1 and 2). The last enzyme, homoserine dehydrogenase, was however, found to be a target of RI-331. The kinetics of inhibition by the antibiotic of homoserine dehydrogenase highly purified from *Saccharomyces cerevisiae* were described here. Homoserine dehydrogenase activity in the forward reaction was inhibited by the addition of NADP into the reaction mixture (Figs. 2 and 3). The possibility that the enzyme-NADP complex may be formed first, followed by interaction with the antibiotic leading to inactivation of the enzyme (Fig. 8), can be supported by the significant inhibition by the antibiotic of the enzyme in the reverse reaction in which enzyme-NADP complex formation is a essential step to reaction (Figs. 5, 6 and 7). The experimental data could be explained if binding of NADP were to induce a conformational change in the enzyme and if this were facilitate binding of the antibiotic, and thus the inactive enzyme-NADP-RI-331 complex could accumulate in the antibiotic-treated yeast cells as the enzyme activity decreases.

A large number of amino acid analogs have been developed as antimetabolites so far, some of which exhibit antifungal activity. The majority of effective compounds have been chemically synthesized, although some were natural products, isolated either as antibiotics or substances toxic to animals[8,9]. We will describe here other amino acid compounds which are possibly available against fungal infections. A recent paper

describes a promising amino acid antifungal antibiotic, cispentacin[10,11] identical to FR109615[12] which is effective in treating candidiasis in mice and has low toxicity to these animals. Moreover, other types of inhibitors which prevent the biosynthesis of essential amino acids other than threonine, isoleucine and methione may also be useful for antifungal chemotherapy, as is RI-331.

Thus, the preferential inhibition by RI-331 of biosyntheses of threonine, methionine, and isoleucine, due to the inhibition of homoserine dehydrogenase (an enzyme not present in animal cells) can account for selective toxicity of the antibiotic against pathogenic fungi.

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